

Chapter 10

Mode of Action on *Trypanosoma* and *Leishmania* spp.



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Abstract In this chapter, the most common molecular targets and mechanisms of action of anti-trypanosomatid drugs are described: biosynthesis of sterols, trypanothione pathway, purine salvage pathway, cysteine proteinases, trans-sialidase, metalloproteases, tubulin, calcium homeostasis and pyrophosphate metabolism, heme uptake and degradation, glycolytic pathway, DNA interaction, oxidative stress and apoptosis. Interaction of the sesquiterpene lactones with heme, the induction of oxidative stress, the inhibition of enzymes as cruzipain and trypanothione reductase, the apoptosis induction and the ability of this type of compounds to inhibit sterol biosynthesis will be also discussed.

Keywords Anti-trypanosomatid drugs · Drug targets · Sterol biosynthesis · Trypanothione pathway · Proteinases · Trans-sialidase · Tubulin · Heme · Oxidative stress · Apoptosis

10.1 Introduction

Among the pathogenic parasites which affect human, trypanosomatids, such as trypanosomes and leishmanias, can be found. These parasites are the causative agents of American trypanosomiasis or Chagas' disease (*Trypanosoma cruzi*), African trypanosomiasis or sleeping sickness (*Trypanosoma brucei*) and leishmaniasis

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(*Leishmania* spp.) The World Health Organization includes trypanosomiasis and leishmaniasis among the group of neglected tropical diseases. These diseases are more prevalent in poor populations, not representing an interesting market for the pharmaceutical industry; efficient vaccines have not been developed; chemotherapy is not always effective, as it presents serious side effects and drug resistance phenomena often occur. Moreover, globalization and migratory currents have favoured the expansion of these diseases into nonendemic zones. Therefore, the need for new and efficient therapeutic and diagnostic alternatives has become evident.

The ideal anti-trypanosomatid drug must attack the parasite with the higher rate of selectivity as possible, due to the fact that not harming or interacting with the host is of great importance to minimize side effects. Molecular structures used as therapeutic agents should show differences with the corresponding analogues in the mammalian host. However, selectivity is not the only parameter that guarantees drug efficiency; it is also needed that the selected target be vital for the parasite. For a target to be validated as such, the drugs (either natural products or drugs designed and optimized *in silico*) that interact selectively with it must show high efficiency.

The knowledge acquired about the basic parasite biochemistry, the application of genetic engineering techniques and the development of bioinformatics and computational techniques are key elements for the identification of these targets. As to *Leishmania* spp. *T. cruzi* and *T. brucei* concern, their respective genomes are known. Therefore, with this information, a comparative genomic analysis with the human genome, or between them, is of great help at the moment of postulating possible therapeutic targets (Jiang and Zhou 2005; Katsila et al. 2016).

10.2 Molecular Targets

Herein, the most common molecular targets for anti-trypanosomatid drugs are described, together with possible mechanisms of action for sesquiterpene lactones (STLs).

10.2.1 Biosynthesis of Sterols

Unlike mammalian cells, which synthesize cholesterol, in trypanosomatids, fungi and yeasts, the sterol synthesis leads to the formation of ergosterol (Fig. 10.1). Sterols (mainly ergosterol and 24-methylsterols) are essential components of the cell membrane. These sterols are not supplied by neither the vector nor the host cell. The sterol biosynthetic route with its inhibitors is schematized in Fig. 10.1.

One of the enzymes on which inhibitors have been tested is squalene synthase (SQS) that catalyses the formation of squalene from two molecules of farnesyl pyrophosphate (FPP). This enzyme is inhibited by two synthetic derivatives of quinuclidine, namely, ER-119884 and E5700 (Tsukuba Research Laboratories, Eisai

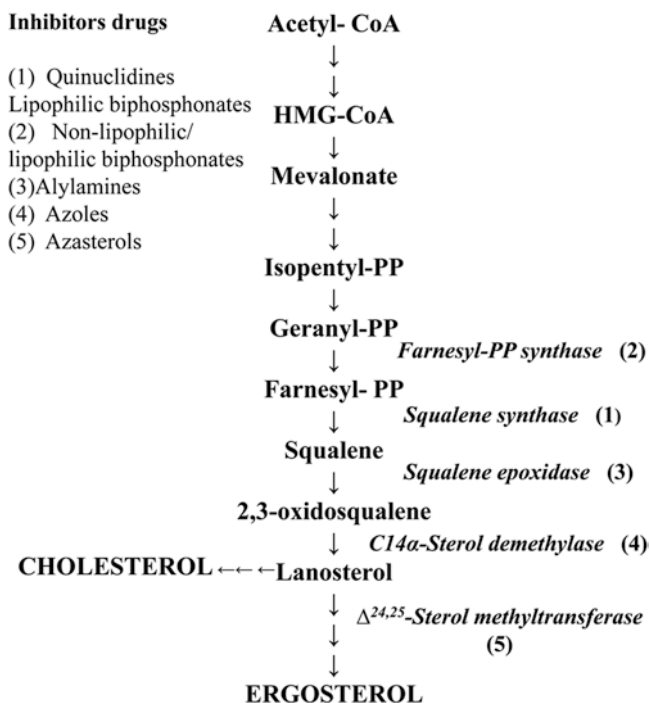


Fig. 10.1 Sterol biosynthetic pathway in trypanosomatids. Reaction steps and the enzymes involved (indicating inhibitory drugs) in ergosterol biosynthesis are depicted. HMG-CoA 3-hydroxy-3-methyl-glutaryl-CoA, PP- pyrophosphate

Co), for which a strong antiparasitic activity against *T. cruzi* (Urbina et al. 2004) and *L. amazonensis* (Fernandes Rodrigues et al. 2008) has been reported. Other important inhibitors of this enzyme are the lipophilic bisphosphonates (Shang et al. 2014), which have also proved to have a considerable inhibitory effect on farnesyl pyrophosphate synthase (FPPS). The usefulness of lipophilic bisphosphonates has been discovered during the process of improving the inhibitory activity of nitrogen-containing bisphosphonate drugs, such as incadronate and ibandronate, which had been reported to be potent inhibitors of human FPPS and SQS (Amin et al. 1992). The nitrogen-containing bisphosphonates have great disadvantages as antiparasitic agents. Firstly, these drugs have a strong binding capacity to human bone mineral (Kavanagh et al. 2006; Mukherjee et al. 2009), and secondly, they are highly polar molecules, which makes the crossing of the plasma membrane to enter the cell difficult.

However, the capacity of lipophilic bisphosphonates to inhibit the synthesis of ergosterol (SQS and FPPS) in more than one point makes them attractive candidates to be evaluated as anti-trypanosomatids drugs. The crystallographic study of *T. cruzi* SQS has allowed not only to carry out a comparative study with human SQS but also to superimpose the molecular structure of ER-119884, E5700 and four representative lipophilic bisphosphonates (BPH-1218, BPH-1237, BPH-1325,

BPG-1344) with that of the enzyme, thus elucidating the structural requirements for these inhibitors to block the active site of SQS (Shang et al. 2014).

Another enzyme belonging to the sterol biosynthetic pathway, for which inhibitors have been tested, is the squalene epoxidase, which converts squalene into 2,3-oxidosqualene. Allylamines, such as terbinafine, are known inhibitors of the enzyme. Antiproliferative effects and ultrastructural alterations induced in vitro by terbinafine on *L. amazonensis* promastigotes and intracellular amastigotes have been reported (Vannier-Santos et al. 1995). The drug is not capable of eradicating the infection by itself, but its activity increases when combined with other inhibitors of the ergosterol pathway. The inhibition of this pathway can also be achieved at the C14 α -sterol demethylase level by azoles, such as ketoconazole or itraconazole, which are effective for the treatment of superficial and systematic mycoses (Buckner 2008; McCall et al. 2015). These commercially available compounds have not been efficient neither in patients nor in animal models of *T. cruzi* infection (McCabe 1988; Moreira et al. 1992; Brener et al. 1993); however, when combined with other drugs, their efficiency could be enhanced. The combination terbinafine with ketoconazole increases almost a hundredfold the activity of terbinafine alone (Vannier-Santos et al. 1995). Moreover, benznidazole, when combined with itraconazole, is more efficient than benznidazole alone in eliminating parasites from the blood. The combination, thus, allows reducing the benznidazole dosage notably (Assíria Fontes Martins et al. 2015). Amiodarone and itraconazole have also shown a synergistic activity (Paniz-Mondolfi et al. 2009). Likewise for azoles, which interact at the C14 α -sterol demethylase level, a series of triazole derivatives have shown great antiparasitic potency. The latter group of drugs includes posaconazole (SCG56592), ravuconazole (BMS 207, 147) and TAK-187, among others (Urbina 2001; Buckner 2008). These compounds are capable of inducing radical parasitological cure both during acute and chronic infections caused by *T. cruzi*. Besides, these drugs are active orally, and they exert little or no toxic effects in mammal cells and are active against nitrofuran- and nitroimidazole-resistant *T. cruzi* strains (Urbina 2010). In humans, posaconazole has shown a considerable synergistic effect with quinuclidine E5700 (Shang et al. 2014), amiodarone (Veiga-Santos et al. 2012) and with benznidazole. Two clinical trials, CHAGASAZOL and E1224, were carried out to analyse the effect of posaconazole and ravuconazole, respectively. In both cases these drugs were compared to benznidazole. These trials demonstrated that azoles are not effective as monotherapy for the treatment of patients in the indeterminate phase of Chagas' disease, unlike benznidazole, which proved to be an efficacious drug to maintain sustained clearance of the parasite even 1 year later (Chatelain 2015).

The enzyme $\Delta^{24,25}$ -sterol methyltransferase is involved in the last steps of the biosynthetic ergosterol pathway. This enzyme is present in trypanosomatids but not in the mammalian host, which renders it a potential therapeutic target. Although azasterols are inhibitors of this enzyme, it has been demonstrated that, though these compounds have suppressor effect, they are ineffective to cure and to prevent disease progression (Urbina 2010).

The interference with the synthesis of sterols is a very interesting therapeutic target. The interaction of drugs with ergosterol can be detrimental to the parasite, since this interaction brings about an alteration of cell membrane permeability, causing the loss of small ions, mainly K^+ and cell death. This mechanism of action would account for the antileishmanial effect of amphotericin B. Miltefosine would exert its effects by interacting with phospholipids and membrane sterols (Silva-Jardim et al. 2014).

10.2.2 Trypanothione Pathway

The trypanothione (N1,N8-bis(glutathionyl)-spermidine) is the main low molecular weight thiol that is exclusively present in trypanosomatids. This thiol in an equilibrium between its oxidized and reduced forms, TS_2 and $T(SH)_2$, respectively, plays a key role in maintaining the intracellular redox state (Manta et al. 2013). Being the trypanosomatids' aerobic organisms, they are exposed to oxidative and nitrosative stress originated from the host and parasite cellular metabolisms. The trypanothione thiol groups play a key role in the parasite's antioxidative defence system. The trypanothione-based redox metabolism provides the reduction equivalents for both detoxification of peroxides by the tryparedoxin peroxidase and ascorbate peroxidase and for the biosynthesis of deoxyribonucleotides by the ribonucleotide reductase (Leroux and Krauth-Siegel 2016). The intracellular levels of TS_2 and $T(SH)_2$ are regulated by the activity of two enzymes, the trypanothione synthetase (Try-S), which is the enzyme that catalyses the synthesis of trypanothione disulphide (TS_2), and the trypanothione reductase (Try-R), which catalyses the NADPH-dependent reduction of TS_2 to $T(SH)_2$. In kinetoplastids, the trypanothione/Try-R system performs functions that are equivalent to the glutathione/glutathione reductase system in mammals. Since both Try-S and Try-R are specific for trypanosomatids and essential for their multiplication, they are promising targets for the development of selective inhibitors.

Kinetic studies performed on Try-S from different trypanosomatids, along with data obtained from structural analyses, have shown the low specificity of the enzyme to spermidine. This finding allows postulating the use of polyamines, which are analogous to spermidine, as inhibitors of this enzyme (Leroux and Krauth-Siegel 2016). Regarding the identification of potential inhibitors, the use of the high-throughput screening techniques has allowed to analyse compound libraries with the purpose of developing lead molecules targeting Try-S (Leroux and Krauth-Siegel 2016).

As for Try-R, three kinds of inhibitors have been considered: competitive inhibitors, irreversible inhibitors and subversive substrates. The tridimensional structure of this enzyme in its three stages (free enzyme and enzyme-substrate and enzyme-inhibitor complexes) is known and has allowed the rational design of drugs which behave as Try-R inhibitors. In addition, the differences existing between the active sites of Try-R and glutathione reductase allow the design of inhibitors that are

specific for one of them (Steenkamp 2002). Many non-structurally related compounds have shown inhibitory activity, i.e. polyamine derivatives, tricyclic aromatic compounds, amino diphenyl sulphides, peptidic derivatives and (terpyridine) platinum (II) complexes (Leroux and Krauth-Siegel 2016; Steenkamp 2002; Chawla and Madhubala 2010; Sueth-Santiago et al. 2017). Even if these compounds act as possible Try-R inhibitors, the antiparasitic activity that they have displayed correlated poorly with the inhibitory potency against Try-R. This phenomenon could be due either to the parasite being able to survive with only 10% of the enzyme activity, to limited uptake of the drug into the parasite, or to the fact that, in vivo, the inhibitor showed affinity for another target, rather than Try-R (Leroux and Krauth-Siegel 2016).

Finally, Try-S and Try-R are not the only trypanothione pathway targets; the enzymes involved in the synthesis of spermidine such as ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (AdoMetDC) are also considered interesting for the development of new drugs against trypanosomiasis and leishmaniasis (Heby et al. 2007).

10.2.3 Purine Salvage Pathway

Trypanosomatid parasites lack the enzymes necessary for the de novo synthesis of purines; therefore, they depend on the salvage pathway of purines to synthesize purine nucleotides from purine bases from the mammalian host (Fig. 10.2). In this sense, both the transport mechanisms of purine bases into the parasite cell and the enzymes of the salvage route become attractive targets to kill the parasite. Although the transporters of both the bases and their nucleosides are different from those of the host in terms of specificity, the multiplicity of transporters present in trypanosomatids (Chawla and Madhubala 2010) makes it difficult to efficiently block them to cause parasite death. As for the enzymes involved in the purine salvage pathway

Fig. 10.2 Enzymes involved in the purine salvage pathway. PRPP phosphoribosyl pyrophosphate

- 1) **Adenine phosphoribosyl transferase (APRT)**
Adenine + PRPP \rightarrow AMP + Ppi
- 2) **Hypoxanthine-guanine phosphoribosyl transferase (HGPRT)**
Hypoxanthine + PRPP \rightarrow IMP + PPI
Guanine + PRPP \rightarrow GMP + Ppi
- 3) **AMP deaminase**
AMP \rightarrow IMP + NH₃
- 4) **Adenosine deaminase**
Adenosine \rightarrow Inosine + NH₃
- 5) **Nucleoside quinase**
Adenosine + ATP \rightarrow AMP + ADP
Guanosine + ATP \rightarrow GMP + ADP
Inosine + ATP \rightarrow IMP + ADP

(Fig. 10.2), they have been identified and found to differ significantly from those of the host, basically as specificity towards the substrate concerns. Allopurinol (hypoxanthine analogue) is an inhibitor of the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) which has shown antiparasitic activity against *Leishmania* and *T. cruzi* (Maya et al. 2007; Raviolo et al. 2013). Allopurinol is phosphorylated by the HGPRT to be incorporated into the nucleic acids as a nonphysiological nucleotide, thus disrupting the synthesis of nucleic acids and the synthesis of proteins, leading to parasite death. Phthalic anhydride derivatives and phthalimide can also be used as structural analogues of purine bases. As trypanosomatids have many alternative pathways for the salvage of purines, the enzymes involved in this process are not essential for parasite survival; therefore, either the simultaneous blockade of more than one enzyme or the combined treatment with other antiparasitic drugs could be effective as therapeutic alternative.

10.2.4 Cysteine Proteinases

In trypanosomatids, the cysteine proteinases, which are homologous to mammalian cathepsins, are the most characterized enzymes. They have become an interesting therapeutic target not only because they are structurally different from their homologues in mammals but also for their role in the host-parasite interaction as putative virulence factors.

Cruzipain is a cathepsin L cysteine proteinase present in *T. cruzi*. This enzyme is encoded by a gene whose expression is under different regulatory mechanisms in the different parasite stages suggesting specific functions for the regulation in each stage (Alvarez et al. 2012). Furthermore, the location of this enzyme varies with the stage, being located in reservosomes, lysosomes and the cell surface and in epimastigotes, trypomastigotes and amastigotes, respectively (Sueth-Santiago et al. 2017; Alvarez et al. 2012). The first designed inhibitors were peptides capable of binding irreversibly to the enzyme, such as diazomethylketone, vinyl sulfone and fluoromethylketone derivatives (Kerr et al. 2009). Non-peptidic inhibitors have also been developed, such as cyclic thiosemicarbazones, nitrile-based inhibitors, benidipine and clofazimine (Ferreira et al. 2010; Caputto et al. 2011; Sbaraglini et al. 2016; Burtoloso et al. 2017). Cruzipain inhibitors have been used in murine models of chronic and acute infections, obtaining parasitological cure with minimum toxicity; however, high doses were required due to their short half-life.

An analysis of the *Leishmania major* genome has shown the presence of about 65 cysteine proteinases, some of which are of the cathepsin L type and others of the cathepsin B type, which are involved in the host-parasite interaction (Chawla and Madhubala 2010). Moreover, a natural inhibitor of cysteine proteinases from *L. mexicana* has been characterized and has proved to be a potent inhibitor of cathepsin B cysteine proteinase. BALB/c mice infected with mutants overexpressing such inhibitors were able to resolve the infection faster than the control group infected with wild-type parasites (Bryson et al. 2009).

In *T. brucei*-infected mice treated with carbobenzoxy-phenylalanyl-alanine diazomethylketone, (Z-Phe-Ala-CHN₂), which is a cathepsin B-cysteine proteinase inhibitor, it is observed that this inhibition depletes the parasite of essential nutrients necessary for DNA synthesis, preventing the progression of the cell cycle (Scory et al. 2007). This *T. brucei* cysteine proteinase has been demonstrated to have differences with its mammalian counterpart, thus being a promissory target for drug design (Kerr et al. 2010).

10.2.5 *Trans-sialidase*

Trans-sialidase (TS) was identified in *T. cruzi* three decades ago. This enzyme is expressed in the trypomastigote form; it is located on the external surface of the parasite and is anchored as a non-integral membrane protein to glycosylphosphatidylinositol, which promotes its secretion to the extracellular environment to act on specific phospholipases. Unlike classic sialidases that hydrolyse sialic acid residues of glycoproteins and/or glycolipids, the TS catalyses the transference of sialic acid residues between glycoconjugates. Sialic acid is not produced by the parasite, and it is one of the most important sugars in the parasite recognition of the mammalian cell. In trypomastigotes, TS allows the parasite to transfer sialic acid from the host cell to its own. In this way, the parasite is no longer recognized as a foreign agent and can then infect host cells without triggering the immune response (Dc-Rubin and Schenkman 2012; Miller and Roitberg 2013). It is known that decreased levels of TS expression could contribute to the loss of *T. cruzi* trypomastigote virulence (San Francisco et al. 2017). Since this enzyme plays a crucial role in parasite survival, a large number of genes encoding highly related but enzymatically inactive proteins are present in the parasite's genome. These proteins, which are expressed simultaneously, would serve to neutralize both antibodies and inhibitors directed to TS. This fact, added to the multiple roles that TS plays in both the biology of the parasite and in the development of Chagas' disease, renders TS difficult to inhibit. The inhibitors tested so far have proved to be weak and non-specific, with high inhibition constants that were in the millimolar order. Inhibitors of the enzymes that hydrolyse sialic acid (sialidase), sialic acid derivatives (compounds that covalently bind to Tyr342 present in the active site of the enzyme), sugar derivatives (such as lactitol, which competes with sialic acid) and sialic acid analogues capable of inhibiting their transfer were tested as inhibitors, but none of them have shown good activity (Sueth-Santiago et al. 2017). Currently, research works are still ongoing to find new chemical scaffolds to inhibit TS.

10.2.6 *Tubulin*

Tubulin is a protein that forms microtubules, which are cytoskeletal filaments that are responsible for maintaining the main functions of eukaryotic cells. Such functions include the segregation of chromosomes during cell division, the transport of intracellular components and the maintenance of the cell shape, cell motility and distribution of plasma membrane components (Sueth-Santiago et al. 2017). Tubulin is present in two isoforms, namely, α - and β -tubulin, which polymerize to form a filamentous cylindrical structure called protofilament. The microtubule, which is formed from a protofilament grouping, is a dynamic structure in which polymerization/depolymerization phenomena coexist in equilibrium. Thus, the microtubule size can change to adapt to different situations, such as those arising during the cell cycle. Cell division and parasite motility are highly dependent on the polymerization/depolymerization equilibrium of tubulin and are essential for infection maintenance. In spite of the structural similarity between the tubulins of the different species, different inhibitors have shown a selective recognition. This behaviour would indicate that the small differences existing between tubulins would function as recognition sites for the different inhibitors. Since the parasite proliferation kinetics is comparable to the cancer cell and certain antineoplastic compounds bind to tubulin, they are also expected to display antiparasitic activity. Thus, anti-*T. cruzi* activity has been reported for taxol, curcumin and natural amidepiperins (Baum et al. 1981; Chakraborti et al. 2011; Sueth-Santiago et al. 2016; Freire-de-Lima et al. 2011).

10.2.7 *Homeostasis of Calcium and Pyrophosphate Metabolism*

In trypanosomatids, Ca^{2+} plays an important role in different cellular functions, such as flagellar movement, differentiation, depolarization of microtubules, host cell invasion and immune response evasion mechanisms, such as antigenic variation (Benaim and Garcia 2011). As in other eukaryotic cells, in trypanosomatids, the disruption of Ca^{2+} homeostasis leads to cell death by apoptosis or necrosis. As regards the intracellular regulation of Ca^{2+} , trypanosomatids possess a single mitochondrion that occupies 12% of the total volume of the parasite. In this mitochondrion large amounts of Ca^{2+} are accumulated. In addition, an endoplasmic reticulum Ca^{2+} -ATPase, a plasma membrane Ca^{2+} -ATPase and large amounts of calmodulin are also involved in the regulation of Ca^{2+} levels. Trypanosomatids also have acidocalcisomes, which are organelles that play a role in the bioenergetics of these parasites, acting as the main reservoir of Ca^{2+} ions together with polyphosphates (mostly pyrophosphate). Within the parasite, the pyrophosphate is hydrolysed by pyrophosphatases, constituting an alternative mechanism to the hydrolysis of ATP to obtain energy. Both the hydrolysis of pyrophosphate and the homeostasis of Ca^{2+} are vital

for the parasite and therefore attractive therapeutic targets. Examples of inhibitors of these events are the bisphosphonates (non-metabolizable analogues of pyrophosphate) that inhibit pyrophosphatases and reactions involving pyrophosphate (Galaka et al. 2017), crystal violet and pentamidine that inhibit the plasma membrane Ca^{2+} -ATPase and antiarrhythmic drugs (amiodarone, dronedarone) that destabilize the intracellular Ca^{2+} homeostasis by altering the mitochondrial electrochemical potential and by alkalizing acidocalcisomes (Serrano-Martín et al. 2009; Benaim et al. 2012; Benaim and Paniz Mondolfi 2012; Benaim et al. 2014).

10.2.8 Uptake and Degradation of Heme

Heme is a fundamental molecule for parasite survival that must be acquired from both vertebrate and invertebrate hosts, for the parasite cannot biosynthesize it (Ciccarelli et al. 2007; Tripodi et al. 2011); however, an excessive amount of free heme is known to be toxic for the parasite. Therefore, an efficient control of its uptake, transport and degradation is required in order to avoid the generation of intracellular oxidative stress. Since both excessive and deficient heme levels lead to parasite death, this molecule becomes an important target for the development of antiparasitic drugs.

In trypanosomatids, several proteins involved in the heme uptake and transport have been identified. Although the mechanism involved in heme transport across biological membranes is not fully understood, experimental data indicate the participation of ATP-binding cassette (ABC) transporters in this process (Cupello et al. 2011; Campos-Salinas et al. 2011). In *Leishmania amazonensis*, LHR1, which is a transmembrane protein that mediates the transport of extracellular heme inside the cell, has been identified (Huynh et al. 2012). Syntenic genes with high-sequence identity to LHR1 have been recognized in the genome of several *Leishmania* species, *T. cruzi* and *T. brucei* (Huynh et al. 2012). Merli et al. (2016) have identified and characterized a *T. cruzi* protein (TcHTE) with 55% of sequence homology to LHR1. TcHTE has been found to be located in the flagellar pocket, where the transport of nutrients occurs. As mentioned above, the degradation of heme is also an important event to avoid its cytotoxic effects. In this regard, both *Leishmania* spp. and *T. cruzi* have been demonstrated to have heme oxygenase activity, which transforms the heme into biliverdin, thus accomplishing heme detoxification (Ciccarelli et al. 2007; Cupello et al. 2011; Lechuga et al. 2016).

It is expected that both, heme structural analogues (which compete with heme for its uptake and/or use) and drugs that act as heme-drug complexes (which may inhibit heme degradation), would then display trypanocidal activity. Thus, vitamin B12 (cyanocobalamin) has shown marked antiparasitic activity against the three stages of *T. cruzi* in vitro and on an acute murine model of Chagas' disease (Ciccarelli et al. 2012). Likewise hemin and vitamin B12 would exert its anti-*T. cruzi* effect through the generation of reactive oxygen species.

Table 10.1 Other possible targets to consider for the design of new anti-trypanosomatid drugs

Targets	Mechanism of action	Reference
Glycolytic pathway	Inhibition of glycolytic enzymes: phosphofructokinase, pyruvate kinase and glyceraldehyde 3-phosphate dehydrogenase	Rodenko et al. (2007), Nowicki et al. (2008), Benaim et al. (2014)
DNA topoisomerases I and II	Inhibits of kinetoplast DNA replication	Babokhov et al. (2013)
Metalloprotease	Inhibition of metalloproteases of the M32 family	Alvarez et al. (2012), Frasci et al. (2012), Alvarez et al. (2013)

Other targets considered for the search of new anti-trypanosomatid drugs are summarized in Table 10.1.

10.3 Oxidative Stress and Apoptosis

Oxidative stress and programmed cell death (PCD) arise as consequences of the interaction of drugs with their therapeutic targets.

Oxidative stress emerges from biologic oxidations that generate oxygen-reduction products such as O_2^- , H_2O_2 and/or OH^\cdot , which are known as reactive oxygen species (ROS). These products cause an oxidative damage that the cellular antioxidant defence system must counteract. Trypanosomatids have an antioxidant defence system (Turrens 2004) which differs notably from that of the host cell (Fig. 10.3). In the parasite, trypanothione behaves in a similar way to glutathione in the host cell. Both cell types possess superoxide dismutase, but trypanosomatids lack catalase, the role of which is replaced by peroxidases. These differences render the parasite more sensitive to the action of ROS, in comparison to the host cell. In conclusion, oxidative stress is produced as a response to a redox unbalance that the antioxidant defence system of the parasite cannot compensate. The imbalance caused by an antiparasitic drug could be the result of its intracellular reduction followed by autoxidation (yielding ROS), its interaction with the heme or with other molecules that generate ROS that the parasite cannot metabolize, or acting as a Try-R or Try-S inhibitor, which would decrease the efficacy of the antioxidant defence system.

Different types of cell death may occur in trypanosomatids, such as apoptosis or PCD (Proto et al. 2013). Although the apoptotic pathway in trypanosomatids has very similar features to that of mammalian cells, such as lipid peroxidation, increase in cytosolic Ca^{2+} levels, changes in mitochondrial membrane potential, the release of cytochrome C from the mitochondrion to the cytoplasm and induction of proteases and DNA fragmentation, trypanosomatids lack their classical effectors or regulators like the TNF-related family of receptors, Bcl-2 family members and caspases. However, they possess an endogenous basic machinery constituted by proteins that

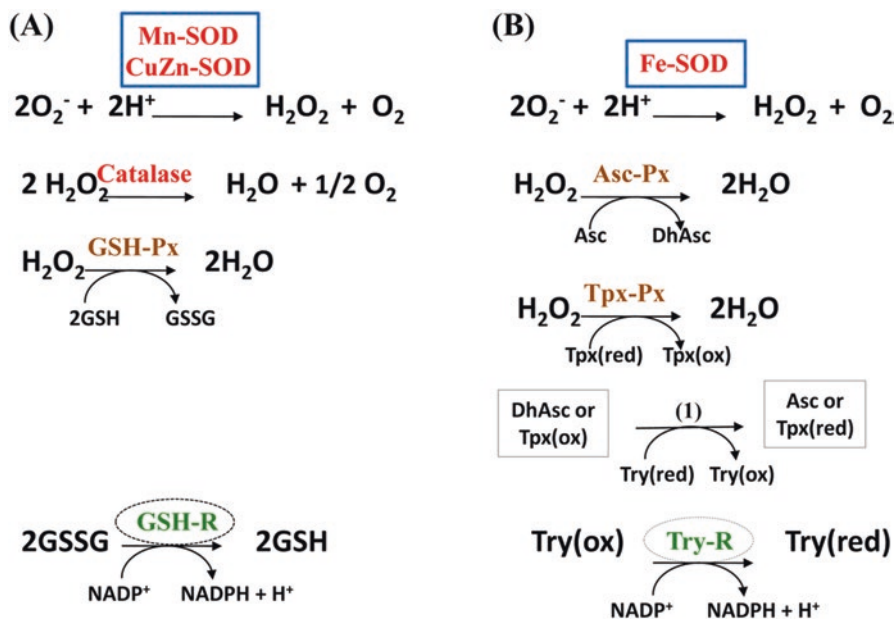


Fig. 10.3 Antioxidant defence enzymatic system in mammalian cells (a) and trypanosomatids (b) GSH glutathione, GSSG oxidized glutathione, Try trypanothione, (ox) oxidized state, (red) reduced state, Asc ascorbate, DhAsc dehydroascorbate, (1) spontaneous reaction, SOD superoxide dismutase, GSH-Px glutathione peroxidase, GSH glutathione reductase, Asc-Px ascorbate peroxidase, Tpx-Px tryparedoxin peroxidase, Try-R trypanothione reductase

control both the cycle and cell differentiation, such as proto-oncogenes, cyclin and cyclin-dependent kinases, which lead to death in a regulated manner. Cell cycle deregulation, heat shock, antiparasitic drugs, nutrient deprivation and ROS are some of the stimuli that can lead to apoptosis in trypanosomatids (Smirlis et al. 2010). Induction of PCD is a particularly important characteristic of antichagasic drugs, due to the fact that apoptosis suppresses the inflammatory response. Taking into consideration that the major cause of myocarditis in chagasic chronic patients is the maintenance of a pro-inflammatory response (Vieira et al. 2012), an antiparasitic drug that induces PCD would not only kill the parasite but also would have a beneficial effect on the host.

10.4 Natural Sesquiterpene Lactones: Parasitic Effects and Probable Targets in Trypanosomatids

Although there are a significant number of reports that describe the anti-trypanosomatid activity of different natural sesquiterpene lactones (Breggio et al. 2000; Schmidt et al. 2002; Jimenez-Ortiz et al. 2005; Saúde-Guimarães et al. 2007;

Table 10.2 Mechanism of action reported for anti-trypanosomatid sesquiterpene lactones

Sesquiterpene lactones	Mechanism	Parasite tested	References
Dehydroleucodine	Intracellular redox imbalance	<i>Trypanosoma cruzi</i>	Brengio et al. (2000)
Mexicanin Helenalin	Intracellular redox imbalance different from dehydroleucodine	<i>Trypanosoma cruzi</i>	Jimenez-Ortiz et al. (2005)
Mexicanin I Dehydroleucodine Psilostachyin	Oxidative stress induction	<i>Leishmania mexicana</i>	Barrera et al. (2013)
Dehydroleucodine Helenalin	Programmed cell death Induction	<i>Trypanosoma cruzi</i>	Jimenez et al. (2014)
Psilostachyin Psilostachyin C	Hemin interaction Ergosterol biosynthesis inhibition	<i>Trypanosoma cruzi</i>	Sülsen et al. (2016)

Sülsen et al. 2008; Karioti et al. 2009; Sülsen et al. 2011; Barrera et al. 2013; Sülsen et al. 2013; Jimenez et al. 2014; Sülsen et al. 2016), little is known about neither their mechanisms of action nor the molecular targets. The mechanisms of action of STLs are summarized in Table 10.2.

Brengio et al. (2000) have demonstrated that the anti-*T. cruzi* effect of dehydroleucodine (DhL) could be blocked by the presence of reducing substrates such as glutathione or dithiothreitol. However, these agents were not able to reverse the effect of DhL if they were added 2 days after the beginning of drug exposure. Based on these results, an intracellular redox imbalance has been proposed to explain the antiparasitic effect of DhL.

The STLs, mexicanin I (Mxn) and helenalin (Hln), have been reported to be equally active against the trypomastigote and epimastigote forms of *T. cruzi* (Schmidt et al. 2002; Jimenez-Ortiz et al. 2005). Since the reducing agents dithiothreitol and beta-mercaptoethanol were not able to reverse the trypanocidal effect of both compounds, it was concluded that Mxn I and Hln are deleterious for *T. cruzi* epimastigotes and that their mode of action would be different than that of the related STL DhL.

To explain the antiproliferative effect of Mxn, DhL and psilostachyin (Psi) on *Leishmania mexicana* promastigotes, Barrera et al. (2013) postulated a direct interaction of the drugs with intracellular sulfhydryl groups. This interaction would alter the non-enzymatic antioxidant defence system, which generates an oxidative stress leading to parasite death. After a short-time treatment (3 h), the induction of oxidative stress would be at least one of the mechanisms of action of DhL, Mxn and Psi, but not for psilostachyin C (PsiC) that would act through another mechanism (Barrera et al. 2013).

Jimenez et al. (2014) have postulated PCD as a possible new therapeutic target. In this sense, they have investigated the induction of PCD in *T. cruzi* by DhL and Hln in comparison with the two conventional drugs, benznidazole and nifurtimox. Both STLs induced PCD in epimastigotes and trypomastigotes, while the conventional drugs did not. This fact could indicate that STLs could act through a different

mechanism of action to kill the parasite. When combined with either benznidazole or nifurtimox, DhL displayed an increased trypanocidal activity. Therefore, the use of both DhL and Hln alone or combined with conventional drugs may be proposed as a potential new therapeutic schedule for the treatment of Chagas' disease.

Artemisinin is an STL that is used as an antimalarial drug, which exerts its activity through heme binding (Schmidt et al. 2012). Taking this phenomenon into consideration, Psi and PsiC, two STLs isolated from plants of the genus *Ambrosia*, have been tested for hemin binding (Sülsen et al. 2016). The results obtained by Sülsen et al. (2016) have shown that both STLs were capable of interacting with hemin, with this interaction leading to an inhibition of heme detoxification and the generation of oxidative stress within the parasite. After a 4-h treatment of *T. cruzi*, Psi induced a fivefold increase in ROS levels. Conversely, PsiC induced a 1.5-fold increase in ROS levels. These results are in agreement with those obtained by Barrera et al. (2013) in experiments performed on *Leishmania mexicana* in which Psi, but not PsiC, in short-time treatment, increased the generation of ROS inside the parasites. Only PsiC was able to inhibit ergosterol biosynthesis, causing an accumulation of squalene upon inhibition of squalene epoxidase. Neither Psi nor PsiC (up to 50 μ M) inhibited cruzipain and Try-R. It can be concluded that despite their structural similarity, both STLs exerted their anti-*T. cruzi* activity through the interaction with different targets. Psi accomplished its antiparasitic effect by interacting with hemin, while PsiC interfered with sterol synthesis. Both STLs induced parasite death by apoptosis. The same type of cell death was observed by Jimenez et al. (2014) for epimastigotes and trypomastigotes of *T. cruzi* treated with DhL or Hln.

Further studies need to be performed in order to characterize the mechanism of action that accounts for the antiprotozoal activity of STLs.

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